Biochem. J. (1986) 240, 107–114 (Printed in Great Britain)

Structural characteristics and intermolecular organization of human pulmonary-surfactant-associated proteins

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The structural relationships and intermolecular organization among the proteins associated with pulmonary surfactant are largely unknown. We studied the pulmonary-surfactant-associated proteins in the bronchoalveolar lavage fluid obtained from a patient with the clinical syndrome of alveolar proteinosis. The major proteins with M_r values of 32000–36000 and 62000 formed thiol-dependent complexes (M_r > 400000) with intermolecular disulphide bonds present in the collgenase-sensitive domains of these proteins. In contrast, other proteins, which were collagenase-insensitive, formed thiol-dependent oligomers that were not covalently linked to the major proteins. The associations of these proteins in the surfactant of a normal individual were similar. By amino acid analysis, two-dimensional peptide mapping and bacterial-collagenase digestion the 32000–36000-M_r and 62000-M_r proteins were nearly identical. Differences in CNBr cleavage products suggested that the larger of the proteins was formed by non-disulphide, covalent, cross-links in the collagenase-sensitive domains of the 32000–36000-M_r proteins. Thus the evidence suggested that the lipid-associated proteins of M_r 32000–36000 contained both disulphide and non-disulphide cross-links in the collagen-like *N*-terminal region of the proteins and form higher-M_r complexes. This organization may support the three-dimensional conformation of surfactant in the alveolar space.

INTRODUCTION

Pulmonary surfactant is a complex mixture of lipids, carbohydrates and proteins which lines the alveolar space (King, 1982; King & Clements, 1972). Its major apparent function is to stabilize alveoli by decreasing surface tension (King, 1974). Phospholipids, such as dipalmitoyl phosphatidylcholine, form a large percentage of the surfactant composition.

There are several non-serum proteins associated with the surfactant phospholipids. The major human pulmonary surfactant-associated protein(s) (PSAP) migrate with apparent-M_r values of 32000–36000 on SDS/polaycrylamide-gel electrophoresis under reducing conditions (Floros *et al.*, 1985). A group of at least eight isoforms containing various amounts of *N*-linked oligosaccharides accounts in large part for the range of M_r displayed by this protein(s) (Ng *et al.*, 1983; Phelps *et al.*, 1984). Cell-free translations of human lung RNA revealed two precursor components with M_r values of 29000–31000 (Floros *et al.*, 1985). A collagen-like domain in the 32000–36000-M_r PSAP is sensitive to bacterial collagenase and contains hydroxyproline (Bhattacharyya *et al.*, 1976; Whitsett *et al.*, 1985). The gene for this protein has been isolated (White *et al.*, 1985), and it codes for 248 amino acids with a predominantly collagen-like sequence of 80 amino acids near the *N*-terminus. Although the function of the PSAP is not entirely clear, it appears to improve the capacity of surfactant phospholipids to reduce surface tension (King & MacBeth, 1979).

In addition to the 32000–36000-M_r protein(s), other non-serum proteins with M_r values of 60000–62000 have been described in human surfactant (Bhattacharyya & Lynn, 1977; Bhattacharyya, 1981; Phelps *et al.*, 1984; Kuroki *et al.*, 1985).

Several of these higher-M_r proteins appear similar to the 32000–36000-M_r PSAP in amino acid and carbohydrate compositions and in antigenic cross-reactivity (Sahu & Lynn, 1979; Kuroki *et al.*, 1985). The precise structural relationship between the 32000–36000-M_r and 62000-M_r PSAP is unclear. The higher M_r of the 62000-M_r PSAP is not due to its covalent association by disulphide bonding with smaller proteins or to glycosylation. Although the 32000–36000-M_r PSAP exists as thiol-dependent complexes in the absence of reducing agents (Whitsett *et al.*, 1985), the possible intermolecular association of this protein(s) with others in the pulmonary surfactant is unresolved.

We studied the PSAP isolated from the alveolar lavage of a patient with alveolar proteinosis. In this disease there is an accumulation of surfactant-like material in the alveoli. Evidence suggests that the PSAP from this alveolar material is structurally identical with that from normal surfactant, but differs in the relative quantity of protein constituents (Floros *et al.*, 1985; Phelps *et al.*, 1984; Whitsett *et al.*, 1985). The major PSAP migrated on SDS/polyacrylamide-gel electrophoresis under reducing conditions with M_r values of 32000–36000 and 62000. These were associated as high-M_r complexes by disulphide bonds located in collagenase-sensitive regions of these proteins. A less-prominent protein with an M_r of 52000 was not covalently associated with the major PSAP, but, in the absence of reducing agents, was present as disulphide-bonded oligomers with an M_r of approx. 180000.

Studies of the isolated 32000–36000-M_r and 62000-

Abbreviations used: PSAP, pulmonary-surfactant-associated proteins; $PhCH_2SO_2F$, phenylmethanesulphonyl fluoride.

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M_r PSAP by amino acid analysis, two-dimensional peptide mapping and bacterial-collagenase digestion revealed striking similarities. The differences in CNBr cleavage products suggest that the 62000-M_r protein is a dimer of the 32000–36000-M_r PSAP. This association, furthermore, appears to be mediated by a covalent cross-linking mechanism that does not involve disulphide bonds.

EXPERIMENTAL

Protein isolation

Alveolar material from an adult male with alveolar proteinosis was a gift of Dr. Karlman Wasserman (University of California, Torrance, CA, U.S.A.). The material had been obtained by bronchoalveolar lavage with sterile saline, at which time the cells were removed by centrifugation at 200 *g*, and the supernatant was freeze-dried and stored at −20 °C. Salt was removed by dialysis against aq. 2.5 mM-EDTA/0.2 mM-$PhCH_2SO_2F$ at 4 °C. The surfactant-containing fraction was recovered as a pellet after centrifugation for 30 min at 48000 *g*. For some experiments water-soluble proteins were removed by washes with distilled water containing the proteinase inhibitors EDTA and $PhCH_2SO_2F$. The water-insoluble pellet was then lipid-extracted by five extractions with chloroform/methanol (2:1, v/v) (Sahu & Lynn, 1979). Reduction and alkylation was performed on a portion of the protein as described by Sage *et al.* (1979).

Separation of the PSAP was achieved by molecular-sieve chromatography on Sephacryl S-200 in 6 M-urea/50 mM-Tris/2.5 mM-EDTA/0.2 mM-$PhCH_2SO_2F$, pH 8.0, at room temperature. Appropriate eluted fractions were pooled, dialysed against distilled water and freeze-dried.

Enzymic and chemical proteolysis

Various PSAP samples were digested with highly purified bacterial collagenase (Advance Biofactures; form III) by the method of Sage *et al.* (1980). Digestions with pepsin (Sigma) were also performed as described by Sage *et al.* (1980). Under these conditions, procollagen type I from cultured human skin fibroblasts was converted into collagen. Proteolytic cleavage with CNBr (Pierce) of PSAP samples was performed as described by Crouch & Bornstein (1978).

SDS/polyacrylamide-gel electrophoresis

Proteins were resolved in discontinuous polyacrylamide slab gels containing 0.5 M urea (Laemmli, 1970). The bands were detected either after staining with Coomassie Brilliant Blue R or after processing for fluorescence autoradiography (Bonner & Laskey, 1974).

Two-dimensional SDS/polyacrylamide-gel electrophoresis was used to evaluate the role of disulphide bonds in the organization of the proteins. Unreduced, lipid-extracted, PSAP were excised from gels after slab-gel electrophoresis. These samples were reduced by 100 mM-dithiothreitol and again subjected to SDS/polyacrylamide-gel electrophoresis in the second dimension.

Peptide mapping

Proteins were also compared by a two-dimensional peptide-mapping technique as described by Elder *et al.* (1977) and further modified for collagens (Sage *et al.*, 1981). Briefly, the PSAP were resolved by SDS/polyacrylamide-gel electrophoresis, and the stained bands were cut from the gel and radioiodinated with Bolton–Hunter reagent (New England Nuclear). The gel slices were incubated for 18 h at 37 °C with 20 μg of proteinase K (EM Biochemicals, Darmstadt, Germany) and the resulting digest was freeze-dried and characterized by thin-layer electrophoresis, followed by t.l.c. on cellulose plates.

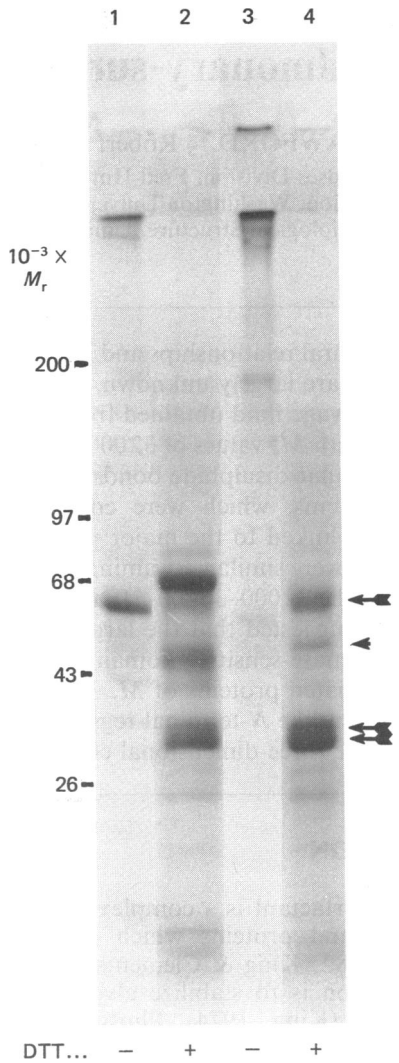

Fig. 1. SDS/polyacrylamide-gel electrophoresis of soluble and insoluble protein isolated from alveolar-proteinosis-patient surfactant

Lavage material recovered as a pellet after centrifugation was lipid-extracted. Water-soluble and -insoluble proteins were freeze-dried and analysed after SDS/polyacrylamide-gel electrophoresis on gradient (4–20%, w/v, acrylamide) slab gels with or without reducing agents. Lanes 1 and 2, soluble proteins; lanes 3 and 4, insoluble proteins. The presence or absence of dithiothreitol (DTT) during electrophoresis is indicated by '+' or '−' respectively. The arrows identify proteins with M_r 62000 (arrow), 32000–36000 (double arrows) and 52000 (arrowhead). The M_r values for protein standards are indicated.

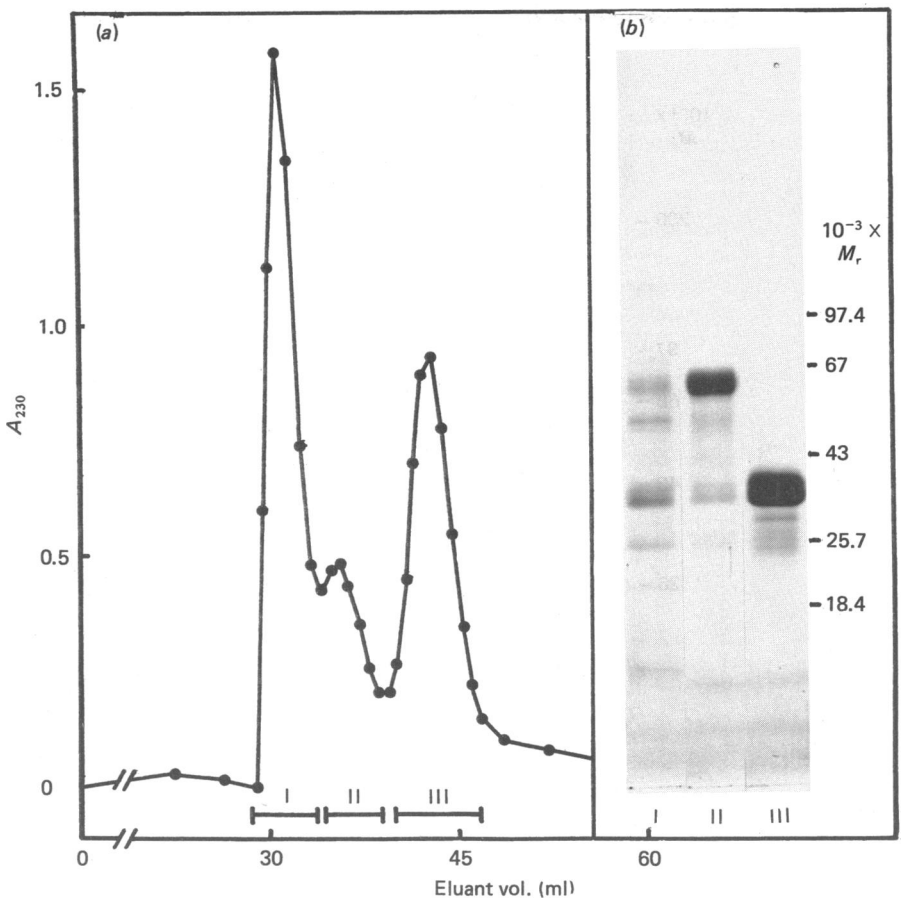

Fig. 2. Molecular-sieve chromatography of PSAP

Samples containing lipid-extracted insoluble protein from alveolar-proteinosis-patient surfactant were reduced and alkylated, and were subsequently chromatographed (*a*) on Sephacryl S-200 in 6 M-urea/50 mM-Tris, pH 8.0, with proteinase inhibitors as described in the Experimental section. Protein was monitored by u.v. absorption at 230 nm. Pooled fractions (indicated by Roman numerals) were dialysed against water, freeze-dried and analysed after SDS/polyacrylamide-gel electrophoresis (*b*). The M_r values for protein standards are indicated.

Immunochemistry

Antiserum to reduced and alkylated PSAP was prepared from a rabbit after subcutaneous injections of 1.0 mg of antigen in incomplete Freund's adjuvant as described by Trueb & Bornstein (1984). Reduced and alkylated PSAP, human serum proteins and human collagen types I, III, IV, V and VI were separated by SDS/polyacrylamide-gel electrophoresis, electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell; 0.45 μm pore size), incubated with the antiserum, followed with ^{125}I-labelled staphylococcal protein A (New England Nuclear; 10^3 c.p.m./ml) and analysed radiographically (Towbin *et al.*, 1979). Fresh human serum was obtained by venipuncture of a normal volunteer and mixed with equal volumes of sample buffer before electrophoresis. Collagen types were isolated after pepsin digestion of human placenta and differential salt precipitations (Sage *et al.*, 1979).

Amino acid analysis

Radiolabelling of reducible cross-linking amino acids with NaB^3H_4 was described by Mecham & Foster (1978). Amino acid compositions of acid- and base-hydrolysed samples were determined by using a Beckman 119C amino acid analyser with a modified program for identifying lysine-derived cross-linking amino acids (Mecham & Lange, 1982).

RESULTS

Protein separation

The migration of water-soluble and insoluble proteins on SDS/polyacryamide-gel electrophoresis before and after thiol-group reduction is shown in Fig. 1. Insoluble PSAP material migrated as high-M_r products under non-reducing conditions. Some of this material migrated with an M_r of approx. 180000. In the presence of dithiothreitol, major bands were of apparent M_r 62000 and 32000–36000 (lane 4, arrows). These bands represented 25% and 52% respectively of the total insoluble PSAP. A less prominent band of M_r 52000 representing approx. 10% of the protein was also present (lane 4, arrowhead). SDS/polyacrylamide-gel electrophoresis of reduced and alkylated PSAP produced the same pattern as that shown in Fig. 1. This pattern was similar to that of PSAP from surfactant isolated by sucrose-gradient centrifugation with the exception of a relative increase in the M_r-52000 band in our samples.

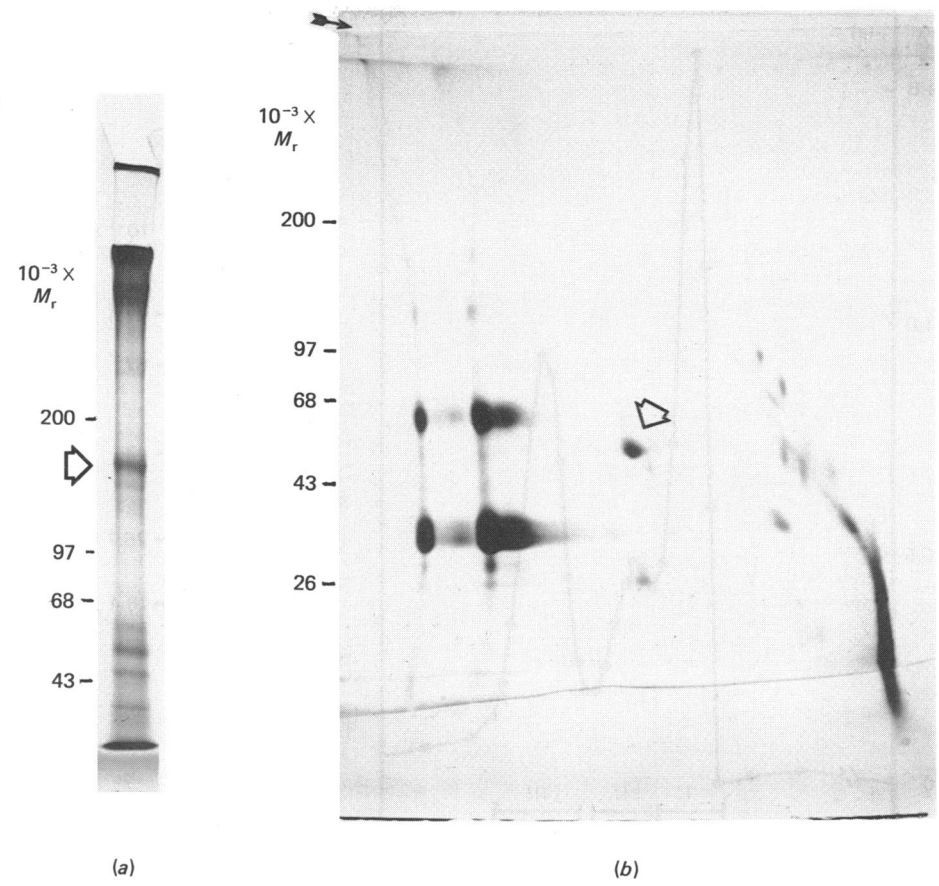

Fig. 3. Two-dimensional electrophoresis of PSAP

Samples containing lipid-extracted insoluble protein from alveolar-proteinosis-patient surfactant were separated by SDS/polyacrylamide-gel electrophoresis on a gradient (4–20%, w/v) slab gel in the absence of reducing agents. The lane containing the protein was excised from the gel, soaked in buffer containing 100 mM-dithiothreitol, and the products were resolved by a second SDS/polyacrylamide-gel electrophoresis on a gradient (10–20%, w/v) slab gel under reducing conditions. (*a*) First-dimension separation of the PSAP under non-reducing conditions; (*b*) second-dimension separation under reducing conditions. The top of the first-dimension gel slice, when positioned horizontally over the second-dimension, is indicated (arrow). The 180000-M_r and 52000-M_r protein bands are indicated (open arrowheads). The M_r values for protein standards are indicated.

There were no differences in the proteins present before and after lipid extraction.

Washing the lipid-extracted material with water effectively solubilized several proteins from the starting material. The water-soluble proteins accounted for approx. 15% of the weight of the starting material and migrated, under reducing conditions, as three major bands with M_r values of 68000, 50000 and 32000–36000 when separated by SDS/polyacrylamide-gel electrophoresis (Fig. 1, lane 2). The band of lowest mobility of these co-migrated with serum albumin, and that of the highest mobility with the 32000–36000-M_r PSAP.

The PSAP that migrated with M_r values of 32000–36000 and 62000 were separated by molecular-sieve chromatography after reduction and alkylation and analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 2). Proteins similar to the starting material in respect of their electrophoretic mobility were eluted with the void volume. Peak II contained the 62000-M_r PSAP and peak III contained the 32000–36000-M_r PSAP (Fig. 2). These PSAP had a tendency to aggregate despite the denaturing conditions. Rechromatography of the proteins eluted in peak III yielded several higher-M_r multimers of the 32000–36000-M_r PSAP (results not shown). This apparent aggregation could be largely inhibited by including 0.1% SDS in the chromatography buffer.

Reduced and alkylated PSAP did not contain contaminating serum proteins. Antiserum raised in rabbits that reacted with PSAP after transfer to nitrocellulose paper after separation by SDS/polyacrylamide-gel electrophoresis did not cross-react with human serum proteins or with collagen types I, III, IV, V or VI (results not shown).

Intermolecular organization

Two-dimensional SDS/polyacrylamide-gel electrophoresis was used to evaluate the role of disulphide bonds in the organization of the PSAP (Fig. 3). The high-M_r bands present after separation of non-reduced proteins were seen to be disulphide-bonded complexes of primarily the 32000–36000-M_r and 62000-M_r proteins. The 180000-M_r component appeared as an oligomer of 52000-M_r protein, which was not disulphide-bonded to the major PSAP (Figs. 3*a* and 3*b*, open arrows). A

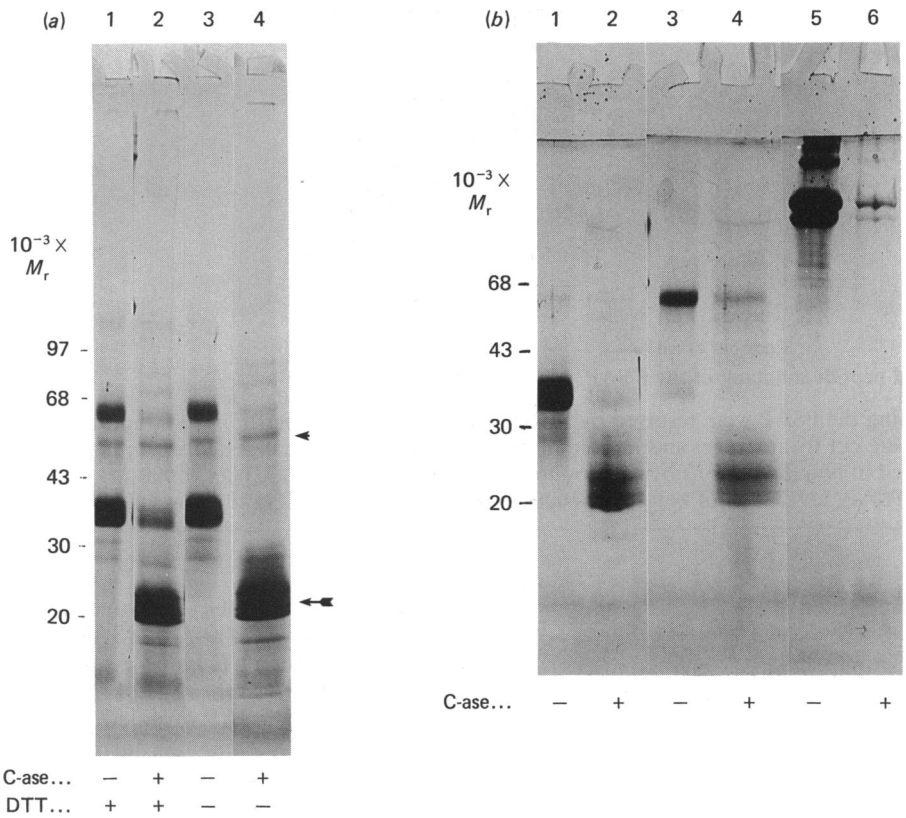

Fig. 4. SDS/polyacrylamide-gel electrophoresis of PSAP after incubation with bacterial collagenase (C-ase)

Samples containing the surfactant-associated proteins from the alveolar-proteinosis patient, before and after reduction and alkylation, were incubated with and without bacterial collagenase. Digestion products were resolved on a gradient (4–20%, w/v) slab gel (*a*) Lanes 1 and 2, PSAP unreduced before incubation; lanes 3 and 4, reduced and alkylated PSAP. The presence of collagenase during incubation is indicated. Reduction by dithiothreitol (DTT) during electrophoretic separation of the products is noted ('+'). The arrowhead indicates a protein with an M_r of 52000 that is not collagenase-sensitive. The solid arrow indicates the peptides generated by collagenase digestion. (*b*) Lanes 1 and 2, 32000–36000-M_r PSAP; lanes 3 and 4, 62000-M_r PSAP; lanes 5 and 6, human collagen type I. The M_r values for protein standards are indicated.

smaller amount of a 28000-M_r protein seen on the gel may also have been associated with this complex.

Disulphide bonding in the water-insoluble protein isolated from normal human surfactant was also examined. Two-dimensional electrophoresis, in the absence and presence of dithiotheitol, resulted in a pattern qualitatively similar to the PSAP from the alveolar-proteinosis patient (results not shown). The relative quantities of proteins in the normal human surfactant appeared different from those of alveolar proteinosis. The normal human surfactant contained relatively less of the 62000-M_r PSAP and more of the 52000-M_r and 28000-M_r proteins compared with surfactant from the patients with alveolar proteinosis.

Digestion of the PSAP with bacterial collagenase was used to examine the location of the intermolecular disulphide bonds in the complex. Analysis by SDS/polyacrylamide-gel electrophoresis of the collagenase-digestion products of unreduced PSAP revealed a series of peptide M_r 20000–25000 (Fig. 4*a*, arrow). The migration of the collagenase-resistant peptides was not altered by the presence of reducing agents in the gel or by reduction and alkylation of the PSAP before digestion (Fig. 4*a*). This result indicates that the intermolecular disulphide bonds were limited to the collagen-like regions of the 62000- and 32000–36000-M_r PSAP. The 52000-M_r protein of the PSAP was not collagenase-sensitive.

Structure of individual PSAP

The 32000–36000-M_r and 62000-M_r PSAP, after isolation by molecular-sieve chromatography, were examined further to assess potential structural similarities between the two. Bacterial-collagenase digestion products of both the isolated 62000-M_r and 32000–36000-M_r PSAP were similar to those generated from the lipid-extracted PSAP as described in Fig. 4(*a*). Both size classes revealed a series of 20000–25000-M_r peptides (Fig. 4*b*).

Two-dimensional peptide mapping, after proteolytic digestion with proteinase K, of the major PSAP was performed on bands excised after SDS/polyacrylamide-gel electrophoresis (Fig. 5). For this experiment, the 32000–36000-M_r PSAP band was separated into upper and lower halves. The two-dimensional peptide maps generated from the 62000-M_r protein were very similar to those from the 32000–36000-M_r protein (Fig. 5). This result suggested that these proteins were structurally homologous.

The amino acid compositions of the 32000–36000-M_r and 62000-M_r PSAP were similar (Table 1). Both contained hydroxyproline and high levels of glycine, but

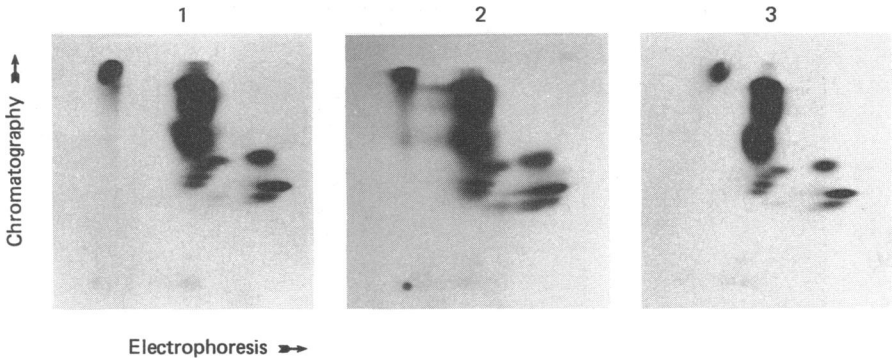

Fig. 5. Two-dimensional peptide mapping of the PSAP

Preparations containing the PSAP were resolved by SDS/polyacrylamide-gel electrophoresis, and the bands corresponding to the major proteins were cut from the gel and radioiodinated. The gel slices were digested with proteinase K and the digestion products were resolved in two dimensions by electrophoresis followed by t.l.c. 1, 32000–36000-M_r PSAP, upper half of band; 2, 32000–36000-M_r PSAP, lower half of band; 3, 62000-M_r PSAP. The origin is in the lower left-hand corner.

Table 1. Amino acid composition of PSAP

Amino acid	Composition (residues/1000 residues) Protein... 32000–36000-M_r	62000-M_r	32000-M_r PSAP*
Cys†	27.8	24.4	30.7
Asx	99.2	112.2	87.7
Thr	52.7	49.3	48.2
Ser	68.2	63.0	48.2
Glx	107.8	117.1	114.0
Pro	63.5	61.1	103.6 §
4-Hyp	22.2	22.8	— §
Gly	155.2	175.8	162.2
Ala	63.9	61.7	57.0
Val	49.8	44.1	43.9
Met‡	14.6	16.2	17.5
Ile	37.6	36.0	39.5
Leu	67.8	76.4	61.4
Tyr‖	26.1	22.0	35.1
Phe	31.1	24.4	26.3
Lys	43.3	33.1	30.7
His	20.7	18.9	21.9
Arg	55.5	49.8	57.0
Trp	ND¶	ND	8.8

* Amino acid composition as predicted by nucleotide sequence studies (White *et al.*, 1985).
† As cysteic acid.
‡ As methionine + methionine sulphoxide.
§ Sum of Pro and 4-Hyp reported as Pro on the basis of nucleotide sequence studies; proline hydroxylation is not detected by nucleotide sequencing.
‖ Not corrected for losses during hydrolysis.
¶ ND, Not determined.

no hydroxylysine. Analysis after borohydride reduction did not reveal the presence of lysinonorleucine, hydroxylysinonorleucine, allysine aldol or the tetrafunctional cross-links desmosine and isodesmosine. The amino acid composition was similar to that calculated by us from the nucleotide sequence of the isolated human PSAP (32000–36000-M_r) gene (White *et al.*, 1985) (Table 1).

In agreement with the data from the amino acid analyses, two-dimensional peptide maps and bacterial-collagenase digests, cleavage of the isolated 32000–36000- and 62000-M_r PSAP with CNBr generated similar fragments (Fig. 6). Analysis after SDS/polyacrylamide-gel electrophoresis of the CNBr cleavage products of both major PSAP revealed broad bands of approx. M_r 25000, 16000 and a narrow band of M_r 10000. However, the 62000-M_r protein had an additional cleavage product of approx. 38000-M_r (lane 4, arrow). Because this fragment may have represented incomplete cleavage secondary to oxidation of methionine residues, samples were reduced overnight with 5% mercaptoethanol at 37 °C before CNBr cleavage for 24 h. This treatment did not alter the resultant peptide patterns.

Pepsin digests

On the basis of the studies noted above, we expected that pepsin digestion of unreduced PSAP would result in release of disulphide-bonded aggregates of collagenase-sensitive peptides. However, repeated pepsin digestions failed to generate new collagenase-sensitive peptides from the lipid-extracted PSAP starting material under conditions that generated collagen from procollagen type I. Similar pepsin treatment of reduced and alkylated PSAP produced peptides of variable M_r. These peptides of 20000–30000-M_r were sensitive to bacterial collagenase (results not shown).

DISCUSSION

Analysis of the intermolecular organization of the PSAP revealed that two of the proteins recovered from the surfactant of the alveolar proteinosis lavage, the 62000- and 32000–36000-M_r proteins, associated as disulphide-bonded aggregates of M_r greater than 400000. Another, 52000-M_r, protein was seen as a disulphide-bonded oligomer of approx. M_r 180000. A 28000-M_r protein also may have been associated with this oligomer. These organizations were confirmed to be present in normal human surfactant.

Digestion of the complex of PSAP with bacterial collagenase, before and after reduction and alkylation, yielded peptides of M_r 20000–25000. This result permitted localization of the intermolecular disulphide bonds of the major PSAP to the collagenase-sensitive region of the proteins. These findings were similar to

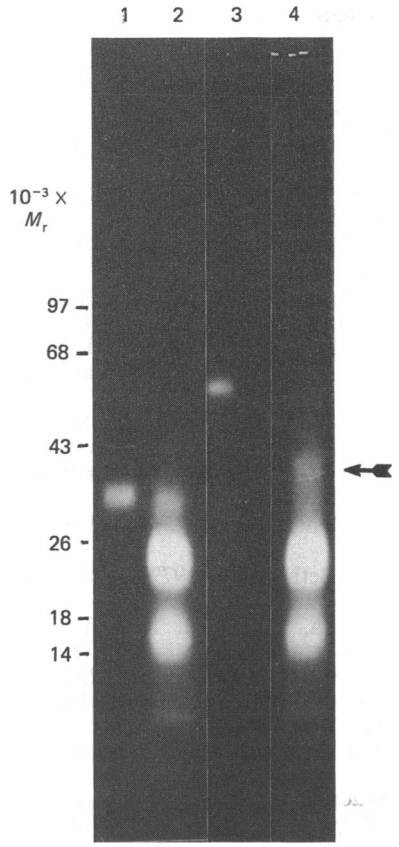

Fig. 6. SDS/polyacrylamide-gel electrophoresis of PSAP after cleavage with CNBr

Samples containing the major PSAP isolated from molecular-sieve chromatography were radioiodinated and cleaved with CNBr. The products were resolved on a gradient (4–20%, w/v) slab gel and revealed by autoradiography. Lane 1, 32000–36000-M_r PSAP starting material; lane 2, 32000–36000-M_r PSAP CNBr cleavage products; lane 3, 62000-M_r PSAP starting material; lane 4, 62000-M_r PSAP CNBr cleavage products. The lanes were reproduced from a contact negative of a photograph of the autoradiogram. M_r values for the protein standards are indicated.

those of Whitsett *et al.* (1985), in which collagenase digestion of a similar protein from normal human lung generated peptides of M_r 20000–22000 that did not form thiol-dependent oligomers.

The failure of pepsin to produce collagenase-sensitive aggregates from non-reduced PSAP was likely due to inaccessibility of the globular regions of the protein. The non-covalent aggregation of reduced and alkylated PSAP during molecular-sieve chromatography in the absence of SDS suggested that there were strong hydrophobic interactions between these proteins. Before reduction the globular regions of the PSAP may therefore have been relatively protected from the aqueous phase by the disulphide-linked collagen-like regions of the proteins and, thus, inaccessible to the pepsin.

The structure of the 52000-M_r protein found in the PSAP was not elucidated. It was not covalently associated with the other major PSAP, but did form disulphide-bonded oligomers. An identity with the 43000-M_r protein in human surfactant described as 'actin' by Postle *et al.* (1985) is unlikely. Its insensitivity to collagenase implies that this 52000-M_r protein is different from one of similar M_r described by Whitsett *et al.* (1985), which had a two-dimensional peptide map similar to that of the 32000–36000-M_r PSAP.

By several criteria the 32000–36000- and 62000-M_r PSAP appeared structurally homologous. Amino acid compositions and collagenase-digestion products were very similar and correlated with the properties predicted from the isolated PSAP gene. Two-dimensional peptide maps of the two major PSAP were also nearly identical. Together, these results suggested that the 62000-M_r PSAP was a dimer of the 32000–36000-M_r proteins. In other species, disulphide-bonded dimers of PSAP exist. A 72000-M_r protein isolated from canine surfactant migrated with an M_r 36000 after reduction (Sueishi & Benson, 1981). The stringent reduction conditions employed during the reduction and alkylation procedure in the present study rendered the presence of disulphide bonding in the 62000-M_r PSAP unlikely. Although Whitsett *et al.* (1985) reported similarities in peptide maps between the 36000-M_r PSAP of alveolar-proteinosis patients and several 'larger molecular weight forms', the latter proteins were not identical in size with the 62000-M_r PSAP described here.

Maps of peptides produced after CNBr cleavage of the major PSAP were consistent with a non-disulphide-linked dimer in the 62000-M_r protein. Although the 32000–36000- and 62000-M_r proteins yielded similar 10000-, 16000- and 25000-M_r peptides, the 62000-M_r protein yielded an additional peptide of M_r 38000. The peptides generated by CNBr cleavage exhibited higher apparent molecular weights than predicted from the positions of methionine residues coded by the PSAP gene. It was not clear whether this anomaly was due to inaccessibility of some methionine residues leading to incomplete digestion, or to structural properties intrinsic to the peptides that produce a lower mobility on SDS/polyacrylamide-gel electrophoresis than predicted from mass alone. Peptides produced after CNBr cleavage of alveolar-proteinosis-patient PSAP described by others appeared closer in estimated M_r to those predicted from the gene structure (Bhattacharyya & Lynn, 1977). However, amino acid composition and *N*-terminal analysis of those peptides did not correlate with the data from gene structure as it is now known. This discrepancy is unexplained, since the amino acid composition described in that study of the 36000-M_r PSAP was nearly identical with the one we report here.

Studies have identified the type II epithelial cell of the lung as the probable site of synthesis of PSAP as well as of surfactant phospholipids. PSAP has been localized, by electron-microscopy/immunocytochemistry, to the rough endoplasmic reticulum, Golgi zone, multivesicular bodies and lamellar bodies in type II cells (Coalson & King, 1984; Walker *et al.*, 1984). These results suggest that a lipoprotein complex is formed before secretion. The PSAP has also been identified associated with intra-alveolar tubular myelin and secondary lysosomes of alveolar macrophages (Williams & Benson, 1981; Sueishi *et al.*, 1977). The association of PSAP with surfactant lipids alters both the structure and activity of the isolated phospholipids (King & MacBeth, 1979; Hawgood *et al.*, 1985). These results are consistent with the insertion of the protein into the lamellae of the lipid

and the resulting lipoprotein complex having domains of lipid in a disordered array (King & MacBeth, 1979).

Cell-free translation of RNA from human and rat lung has confirmed that precursors of the major PSAP have M_r values of less than 31000 and are specific to lung and tissue (Floros *et al.*, 1985, 1986). These findings suggest that the higher-M_r PSAP result from post-translational modification and/or extracellular processing of the primary translation products. These modifications likely include glycosylation and covalent cross-linking in a manner similar to the processing of collagen (Bornstein & Sage, 1980). It is possible that the three-dimensional structure of the lipoprotein complex is influenced by the intermolecular cross-links in the collagen-like *N*-terminal portion of the PSAP, whereas the globular portion is inserted into the lamellae of the lipid. This model is consistent with the appearance of tubular myelin under the electron microscope, which revealed rod-like particles, in regularly spaced rows, which were perpendicular to the bilayered lipid membranes (Hassett *et al.*, 1980).

It is evident that there are several proteins associated with surfactant phosphlipids that may contribute to the function of surfactant and formation of tubular myelin. Some of the PSAP exists as disulphide-bonded complexes. We believe that some of the higher-M_r PSAP are non-disulphide, covalently bonded, dimers of the 32000–36000-M_r glycoproteins. The nature of these cross-links requires further study. Since there is an increase in the amount of tubular myelin in the alveoli of patients with alveolar proteinosis it will be important to correlate the relative increase in the 62000-M_r PSAP with the formation of this surfactant structure (Hook *et al.*, 1978).

We thank Dr. Karlman Wasserman (University of California) and Dr. David Madtes (University of Washington) for providing alveolar-lavage material, Dr. Paul Bornstein and Dr. Rajesh Kapoor (University of Washington) for useful discussions, and Ms. Liz Woodlock for photography. This work was supported in part by National Institutes of Health Grants HL18645, HL07019, GM29853, HL29594 and HL07287. H.S. is an established Investigator of the American Heart Association, with funding contributed in part by the American Heart Association, Washington Affiliate.

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Received 6 May 1986/14 July 1986; accepted 25 July 1986